The Journal of Immunology
RESEARCH ARTICLE | APRIL 01 1980

Functional effects of thymopoietin32-36 (TP5) on cytotoxic lymphocyte precursor units (CLP-U). I. Enhancement of splenic CLP-U in vitro and in vivo after suboptimal antigenic stimulation. FREE

C Y Lau; ... et. al

J Immunol (1980) 124 (4): 1861–1865.
https://doi.org/10.4049/jimmunol.124.4.1861

0022-1767/80/1244-1861$02.00/0
THE JOURNAL OF IMMUNOLOGY

Vol. 124, No. 4, April 1980
Printed in U.S.A.

FUNCTIONAL EFFECTS OF THYMOPOIETIN$_{32-36}$ (TP5) ON CYTOTOXIC LYMPHOCYTE PRECURSOR UNITS (CLP-U)

I. Enhancement of Splenic CLP-U *in Vitro* and *in Vivo* After Suboptimal Antigenic Stimulation

CATHERINE Y. LAU AND GIDEON GOLDSTEIN

From Immunopharmacology, Ortho Pharmaceutical (Canada) Ltd., 19 Green Belt Drive, Don Mills, Ontario M3C 1L9 and The Division of Immunosciences, Ortho Pharmaceutical Corporation, Raritan, New Jersey 08869

Cytotoxic lymphocyte precursor units (CLP-U) were enumerated in the spleens of C57BL/6 mice 3 days after i.p. injections of synthetic thymopoietin$_{32-36}$ (TP5). One hundred to 1000 ng TP5/mouse potentiated splenic CLP-U, this effect being detectable only after suboptimal allogeneic sensitization (with 1.2 × 10^5 mitomycin-C treated DBA cells). This elevation of CLP-U persisted in the injected mice for at least 14 days. Control peptide did not affect CLP-U. *In vitro* incubation of 0.01 to 0.1 ng/ml of TP5 with normal C57BL/6 spleen cells also enhanced CLP-U after suboptimal allogeneic stimulation; high concentrations of TP5 caused suppression of CLP-U and this was detectable with optimal sensitization conditions. Thus TP5, *in vitro* and *in vivo*, appears to regulate immune responsiveness and this regulation varies with TP5 dosage and with the immune stimulus.

Thymopoietin is a polypeptide hormone of the thymus whose 49 amino acid sequence has been determined. By peptide synthesis it has been shown that the pentapeptide arginyl-lysyl-aspartyl-valyl-tyrosine (termed thymopoietin pentapeptide or TP5),[1] corresponding to residues 32–36 of thymopoietin, has the biologic activity of thymopoietin in a number of assays (1, 2), and thus probably corresponds to an active site of the native molecule.

The biologic activity of thymopoietin has been studied most closely in relation to its activity in inducing early T cell differentiation and inhibiting B cell differentiation (3–6). These studies have mainly focused on the effects of thymopoietin in inducing phenotypic changes in cell surface antigens that correspond with T cell differentiation; a cyclic AMP second signal was implicated in the transmission of the inductive signal in prothymocytes. More recently, it was found that thymopoietin affected the function of more mature peripheral lymphocytes and that a cyclic GMP signal was likely involved in these actions (1, 7). Our present studies were directed at the functional effects of TP5 in normal thymus-intact mice. Evaluation of cytotoxic lymphocyte precursor units (CLP-U) utilizing a limiting dilution technique was employed as a sensitive indicator of immunologic responsiveness. TP5, *in vitro* and *in vivo*, clearly enhanced the frequency of CLP-U, but this effect was only detected under conditions wherein antigenic stimulation was suboptimal. By contrast, TP5 in higher amounts *in vitro* and *in vivo* appeared to suppress the enumeration of CLP-U.

Received for publication September 17, 1979.
Accepted for publication January 10, 1980.
The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] Abbreviations used in this paper: CLP-U, cytotoxic lymphocyte precursor units; TP5, thymopoietin pentapeptide; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

MATERIALS AND METHODS

Mice. Female, 8-week-old, C57BL/6 (H-2^b) mice and DBA/2J (H-2^d) mice were purchased from the Jackson Laboratory, Bar Harbor, Maine.

Peptides. TP5 (arginyl-lysyl-aspartyl-valyl-tyrosine) was synthesized as described previously (2). A control peptide (tyrosyl-arginyl-lysyl-aspartyl-valine) was similarly synthesized.

Medium. The medium used for cell cultures in all the experiments was RPMI 1640 (Gibco, Grand Island, N. Y.), to which was added 10% fetal calf serum (Gibco, Cat. No. CA483218), 5 × 10^{-5} M 2-mercaptoethanol and 10 mM HEPES buffer.

TP5 treatment in vitro. Single-cell suspensions were made from the spleens of normal C57BL/6 mice and the cells were washed three times with PBS. The cells were then resuspended in RPMI medium without any additions at 10^7/ml and then incubated with various concentrations of TP5 for 1 hr at 37°C. After incubation, the cells were washed and placed in culture as described below.

Limiting dilution assay. The frequency of CLP-U was estimated by the limiting dilution assay according to the method of Teh *et al.* (8) and Lindahl and Wilson (9). Limiting numbers of C57BL/6 spleen cells (2 × 10^4 to 8 × 10^4) were put in V-bottom Linbro trays (Flow Laboratories, Cat. No. 76-024-05) together with mitomycin-C-treated DBA spleen cells (10^7 DBA cells treated with 30 μg of mitomycin-C for 30 min at 37°C). The number of DBA cells used varied from 1.2 × 10^5 to 6.5 × 10^5 per culture. Twenty replicates were set up for each cell concentration. The cultures were incubated at 37°C for 6 days. At the end of the incubation period, 100 μl of the supernatant medium were removed from each well, and the cells were then mixed with 0.1 ml of fresh medium containing 2 × 10^4 P815 mastocytoma cells labeled with ^{51}Cr as described previously (8). After a further incubation period of 4 hr, 100 μl of the supernatant fluid in each well were removed and radioactivity was determined in a gamma spectrometer. The background chromium release from cultures containing only C57BL/6 cells or DBA cells was always less than 15%.

Calculations. The frequencies of CLP-U were calculated

according to Poisson's statistics. The mean spontaneous release was calculated by averaging the counts of 20 wells that received only C57BL/6 cells. Wells were scored as positive if their counts were greater than 2.07 standard deviations above the mean of the control ($p < 0.05$). According to Poisson's distribution, the frequencies of precursors can be calculated by the equation:

$$p = e^{-\delta N} \quad (1)$$

where p = probability of response
δ = frequency
N = number of cells put in culture

The percent of nonresponding cultures for each C57BL/6 concentration was calculated and plotted on semi-logarithmic scale as a function of the cell concentration. According to equation (1), the inverse of the number of cells in culture that will give 37% nonresponding culture is the frequency of precursors in that culture. The best fit regression line by least square method was calculated for each line and the frequency was then estimated.

Mixing experiments. Spleen cells from C57BL/6 mice were incubated with various concentrations of TP5 *in vitro* and then washed as described above. These treated cells were then mixed in 1:1 ratio with normal untreated C57BL/6 spleen cells and the resultant mixed cultures were then adjusted to the required concentrations and set up for limiting dilution assays as described above.

RESULTS

Effect of TP5 on splenic CLP-U of C57BL/6 mice in vivo. Cytotoxic activity of spleen cells from C57BL/6 mice was generated by allogeneic stimulation with mitomycin-C treated DBA spleen cells and detected by the limiting dilution method. Figure 1 shows semilogarithmic plots of the percent nonresponding cultures as a function of the number of C57BL/6 spleen cells in culture. The frequencies of CLP-U (See *Discussion*) obtained upon stimulation by different numbers of DBA cells were: stimulation with 5×10^5 DBA cells, frequency = 4.3×10^{-5}; stimulation with 2.5×10^5 DBA cells, frequency = 3.0×10^{-5}; stimulation with 1.25×10^5 DBA cells, frequency = 2.0×10^{-6}.

Prior injection of the mice with TP5 affected the frequency of CLP-U that could be enumerated in the spleens of C57BL/6 mice (Fig. 2). Potentiation of CLP-U was only observed with suboptimal stimulation by 1.25×10^5 DBA cells and this was maximal after injection of 10 to 1000 ng/mouse i.p. 3 days before sacrifice (Fig. 2).

Effect of control peptide (Tyr-Arg-Lys-Asp-Val) on CLP-U in vivo (Fig. 3). A control peptide of the same size and charge as TP5 was synthesized and tested in the CLP-U assay in exactly the same way as shown in Figure 2. Figure 3 shows the results of such an experiment; no enhancement or suppression of CLP-U was obtained at any dose level.

Duration effect of TP5 on in vivo CLP-U (Fig. 4). Splenic CLP-U were enumerated at various time intervals after a single injection of either 10 ng, 100 ng, or 1000 ng of TP5. Spleens from the injected mice were set up for limiting dilution assays on the same day. Figure 4 illustrates the prolonged effect of TP5 on CLP-U. Ten nanograms per mouse showed a transient and slight elevation of CLP-U at around day 3 to day 5. One hundred to 1000 ng/mouse sustained an elevated level of CLP-U in the injected mice for at least 14 days.

Effect of TP5 on splenic CLP-U in vitro (Fig. 5). The *in vitro* effect of TP5 on the generation and regulation of CLP-U was studied by exposing the responding cells to TP5 before stimulation in the limiting dilution assay. Enhancement of CLP-U upon suboptimal stimulation was observed in TP5 at concentrations 0.01 to 0.1 ng/ml. TP5 did not enhance CLP-U after optimal stimulation with 5×10^5 DBA but had, in fact, caused suppression of CLP-U in this optimal assay at the higher concentrations of 10 to 100 ng/ml.

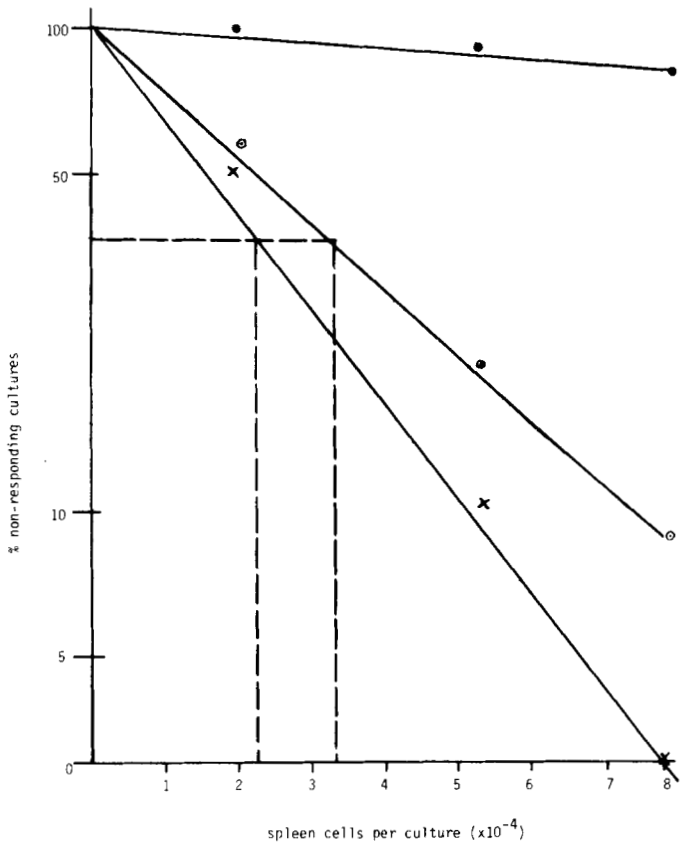

Figure 1. Limiting dilution graph showing the percentage of nonresponding cultures of C57BL/6 spleen cells in a cytotoxicity assay after stimulation by varying numbers of mitomycin-C treated DBA spleen cells. 2.0×10^4 to 8×10^4 C57BL/6 spleen cells were cultured with different numbers of mitomycin-C-treated DBA cells, and 20 replicates were set up for each spleen cell dose. After 6 days, the cultures were assayed against ^{51}Cr P815 cells. Cultures with ^{51}Cr release values less than two standard deviations above the mean of nonstimulated cultures were taken as negative. According to Poisson's statistics, the frequency of CLP-U was calculated from the inverse of the concentration of cells that yielded 37% nonresponding cultures (shown by the heavy broken lines): 5×10^5 DBA cells (×), frequency = 4.3×10^{-5}; 2.5×10^5 DBA cells (⊙), frequency = 3.0×10^{-5}; 1.25×10^5 DBA cells (●), frequency = 2.0×10^{-6} (computer calculation as 37% nonresponder point does not fall on graph).

Effect of TP5-treated cells on regulating CLP-U (Table I). Spleen cells from C57BL/6 mice were incubated with different concentrations of TP5 *in vitro* as described in Figure 5. The cells were washed and then mixed in 1:1 ratio with normal, untreated spleen cells. The mixed population was stimulated with 1.25×10^5 or 2.5×10^5 mitomycin-C treated DBA spleen cells. The frequencies of precursors from both mixed and nonmixed cultures were estimated. Table I compares the observed frequencies of CLP-U values with the expected frequencies (arithmetic mean of the frequencies of the two cultures when stimulated separately). Spleen cells treated with 0.001 to 0.1 ng/ml TP5 had increased frequencies of CLP-U. On mixing

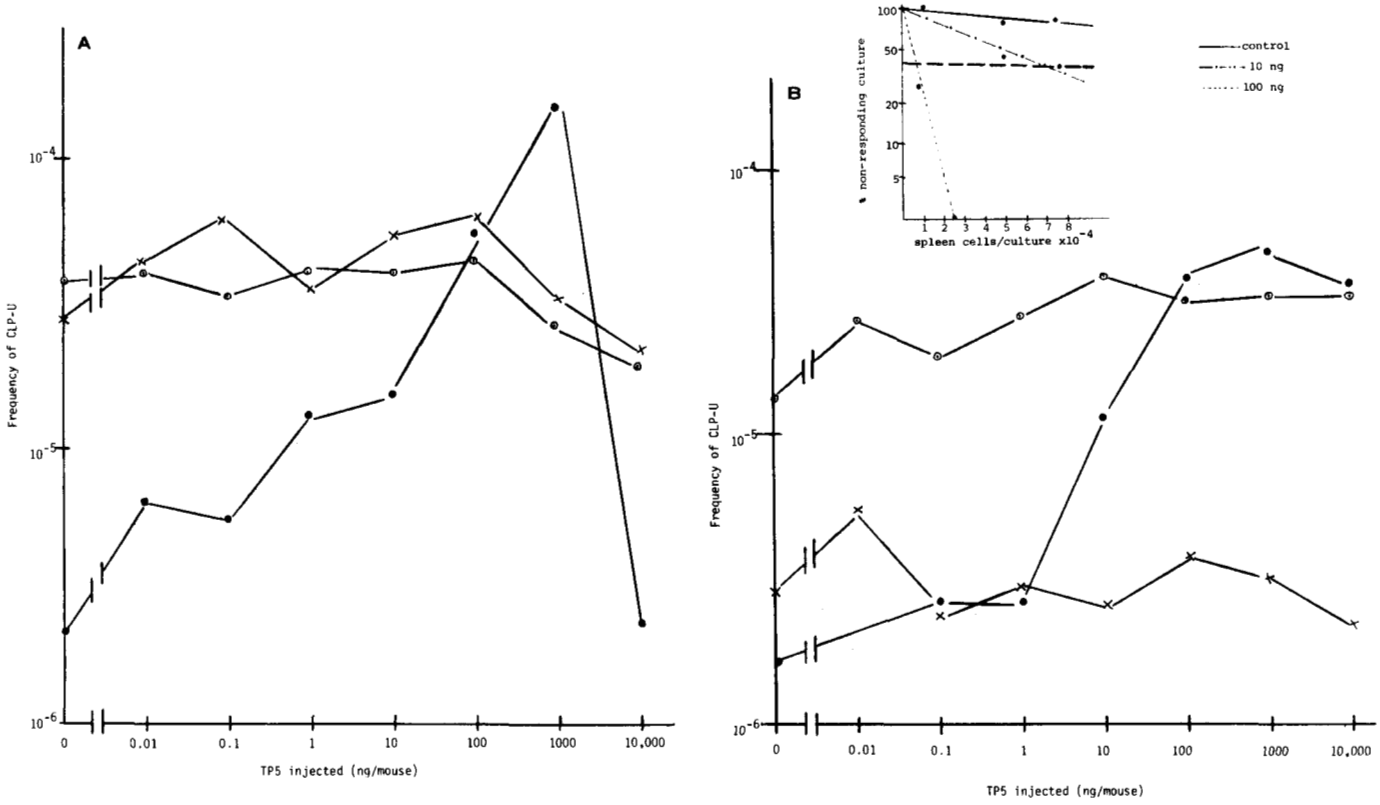

Figure 2. *A* and *B*, graphs showing two representative experiments of the frequencies of CLP-U detected in the spleen of mice injected 3 days previously with varying amounts of TP5. Each line represents the frequency of CLP-U obtained after stimulation by different numbers of DBA spleen cells. The pooled spleens of three mice (six in the control group) were diluted to the appropriate concentrations and then stimulated with varying numbers of DBA spleen cells. The cultures were set up and assayed as indicated in Figure 1. 5×10^5 DBA cells (×); 2.5×10^5 DBA cells (⊙); 1.25×10^5 DBA cells (●). The limiting dilution graph showing the percentage of nonresponding cultures of C57BL/6 spleen cells upon stimulation by 1.25×10^5 DBA cells is shown in the insert. As can be seen from the graph, potentiation of CLP-U by TP5 was most pronounced upon suboptimal (1.25×10^5 DBA cells) allogeneic stimulation.

TP5 treated and untreated cells, the frequencies of CLP-U in the mixed cultures were much higher than expected if there was no interactions. By contrast, spleen cells treated with 10 to 100 ng/ml TP5 showed a slight decrease in frequency of CLP-U, and the mixed cultures showed even lower frequencies than expected.

DISCUSSION

Our present studies focused on the immunoregulatory actions of the synthetic pentapeptide Arg-Lys-Asp-Val-Tyr corresponding to positions 32 to 36 of the sequence of thymopoietin (TP5). The activity of TP5 before sensitization was studied *in vitro* or *in vivo*. We used the limiting dilution technique to enumerate the frequency of CLP-U in C57BL/6 spleen cells after *in vitro* stimulation by mitomycin-C treated DBA spleen cells. The limiting dilution curves for control or TP5-treated mice turned out to be straight lines so the frequency of CLP-U can be estimated based on the 0 order term of Poisson's distribution. This technique was used for a number of reasons: 1) This measures the effect of TP5 on both lymphocytes and ancillary cells since these are all placed together and cytotoxicity is measured in the same well as sensitization. 2) Cytotoxicity of the entire contents of the well is measured rather than effector to target ratios with lymphocytes as is done in conventional measurements of cell-mediated cytotoxicity. Thus, the limiting dilution technique measures effects due to proliferations of cytotoxic effective cells as well as effects due to enhancement of cytotoxic killing and provides a more complete indication of the total effect of the compound on immunoregulation. The frequency of CLP-U obtained clearly did not represent the absolute number of splenic cytotoxic lymphocyte precursors and it is probable that assays used by others also do not since optimal conditions only represent the present "state of the art". Nevertheless, we found that using this technique to define an operational unit provided a useful index for comparing precursor unit contents of various cell populations. 3) *In vitro* sensitization on replicate cell populations permits an accurate measurement of regulation of responses to suboptimal, optimal, and supra-optimal stimulating doses of sensitizing cells.

Using this limiting dilution technique to enumerate splenic CLP-U, we have studied the effect of TP5 in modulating cell-mediated cytotoxicity both *in vivo* and *in vitro*. Intraperitoneal injection of 100 to 1000 ng TP5/mouse 3 days before harvesting spleen cells for *in vitro* assay enhanced significantly the CLP-U under suboptimal allogeneic stimulating conditions but did not affect CLP-U when the sensitization conditions were optimal. No change in the frequency of CLP-U could be observed in mice that had received the control peptide (tyrosyl-arginyl-lysyl-aspartyl-valine). These data suggest that TP5, at these doses, can specifically enhance a suboptimal or defective immune response but does not hyperstimulate a normal response; its action in this regard is quite distinct from classical adjuvants. Because of limitations in *in vitro* culture conditions, CLP-U obtained at supra-optimal stimulation may be reduced (Fig.

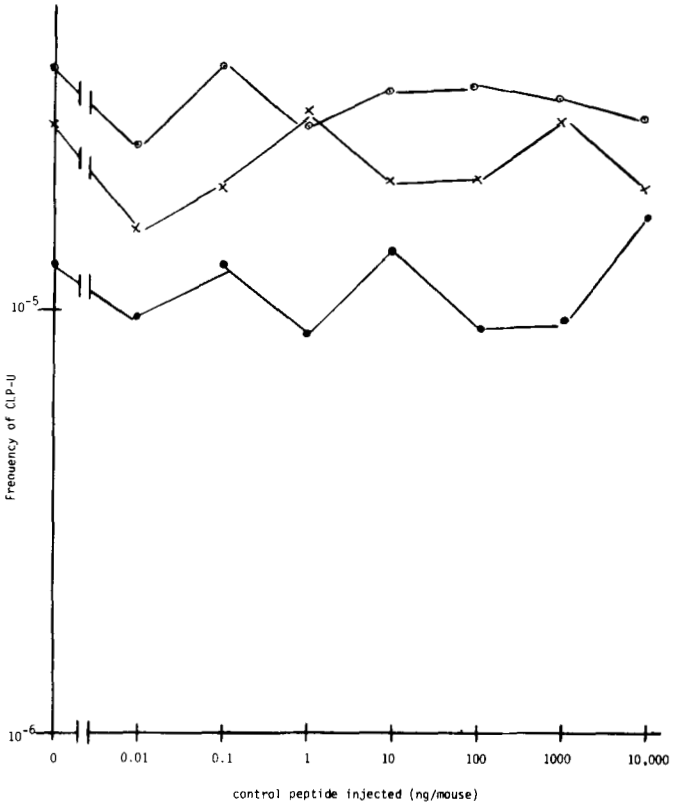

Figure 3. Graph showing the frequencies of CLP-U *in vivo* 3 days after injection with control peptide (Tyr-Arg-Lys-Asp-Val). All experimental conditions were identical to that of Figure 2. No stimulation of CLP-U was observed at any dose studied. 5×10^5 DBA cells (×); 2.5×10^5 DBA cells (⊙); 1.25×10^5 DBA cells (●).

2*b*). Under such circumstances, the effect of TP5 on CLP-U is difficult to predict.

The *in vivo* time studies highlighted the persistence of the effects of TP5. Fourteen days after a single injection of TP5 an enhanced level of immune response to suboptimal antigenic sensitization was still maintained. Since metabolic studies indicate that TP5 has a half-life of approximately 30 sec in plasma (10) it appears that TP5 rapidly triggers events in the immune system and that these perturbations persist for several weeks in the absence of further TP5.

In vitro incubation of 0.001 to 1.0 ng/ml of TP5 with C57BL/6 spleen cells also augmented significantly the CLP-U generated by subsequent stimulation with suboptimal numbers of mitomycin-C treated DBA spleen cells (1.25×10^5, 2.5×10^5 cells), this effect being comparable with the data obtained with *in vivo* injections. Higher concentations of TP5 (10 to 100 ng/ml), however, inhibited CLP-U under optimal stimulating conditions (5×10^5 cells). These results suggest a regulatory role of TP5 in modulating the immune response. This regulatory effect of TP5 was studied in a mixing experiment. Spleen cells that were incubated with 0.01 to 0.1 ng/ml of TP5 could enhance CLP-U from syngeneic spleen cells that had never been exposed to TP5. This syngeneic enhancement was greater in the case of suboptimal stimulation, further suggesting the regulatory role of these TP5-treated cells. Ten to 100 ng/ml of TP5 showed a suppression of the frequency of CLP-U after optimal stimulation and these cells suppressed the response of normal cells under similar conditions. These results suggest that TP5 can regulate the immune response by acting on the regulatory subpopulation of cells.

Our findings with TP5 can be extended by implication to thymopoietin itself, since TP5 has the biologic activity of the native molecule. These findings carry a number of implications that will require substantiation with further experiments. Firstly, thymopoietin appears to regulate the development of cell-mediated cytotoxicity by predominantly affecting regula-

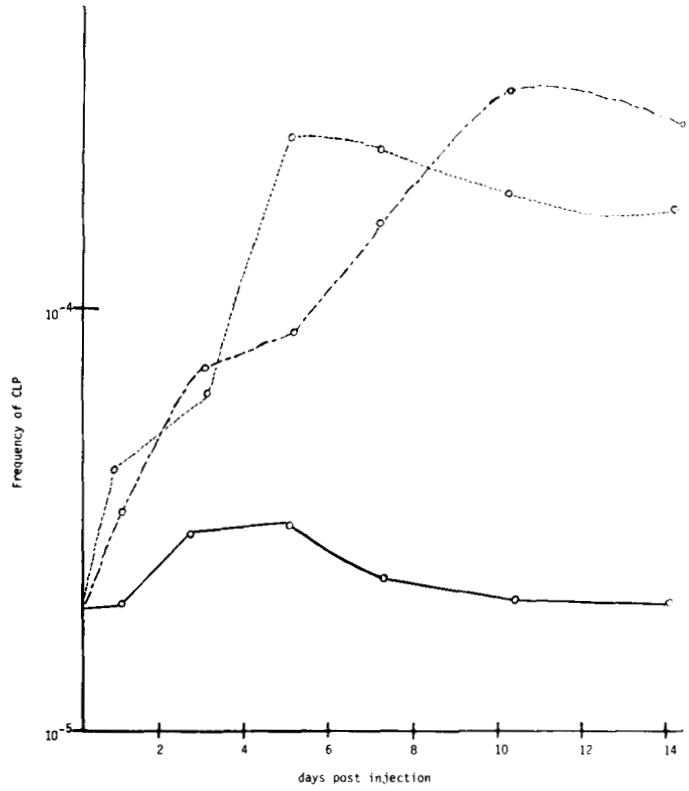

Figure 4. Graph indicating the effect of a single injection of TP5 on the frequency of CLP-U enumerated with suboptimal allogeneic stimulation (1.25×10^5 DBA cells). Groups of three C57BL/6 mice (six in the control) were injected with 10 ng (O——O), 100 ng (O-----O), or 1000 ng (O-----O) of TP5 on different days before the experiment. Limiting dilution assays were set up for all these groups on the same day (see Figure 1 and *Materials and Methods*). TP5, 10 ng/mouse, showed slight and transient enhancement at days 3 and 5 whereas TP5, 100 ng and 1000 ng/mouse, showed a prolonged effect on the frequency of CLP-U that was still detectable 14 days after injection.

TABLE I

Comparison between the expected and observed frequencies of CLP-U upon mixing TP5 treated and untreated spleen cells

	Frequency of CLP-U after Stimulation with 1.2×10^5 DBA Cells ($\times 10^{-7}$)				Frequency of CLP-U after Stimulation with 2.5×10^5 DBA Cells ($\times 10^{-7}$)			
TP5 concentration	Preincubated with TP5	No preincubation	Expected values[a] from mixed cultures	Observed values from mixed cultures	Preincubated with TP5	No preincubation	Expected values[a] from mixed cultures	Observed values from mixed cultures
ng/ml								
0	3	5	4.0	4.5	80	90	85	87.5
0.001	5	5	5.0	340	80	90	85	230
0.01	14	5	9.5	250	260	90	175	450
0.1	14	5	9.5	34	223	90	160	340
10	5	5	5.0	1	100	90	95	90
100	1	5	2.5	1	68	90	79	50
1000	1	5	2.5	1	34	90	62	20

[a] Arithmetic mean of the values in column I (preincubated with TP5) and column II (no preincubation).

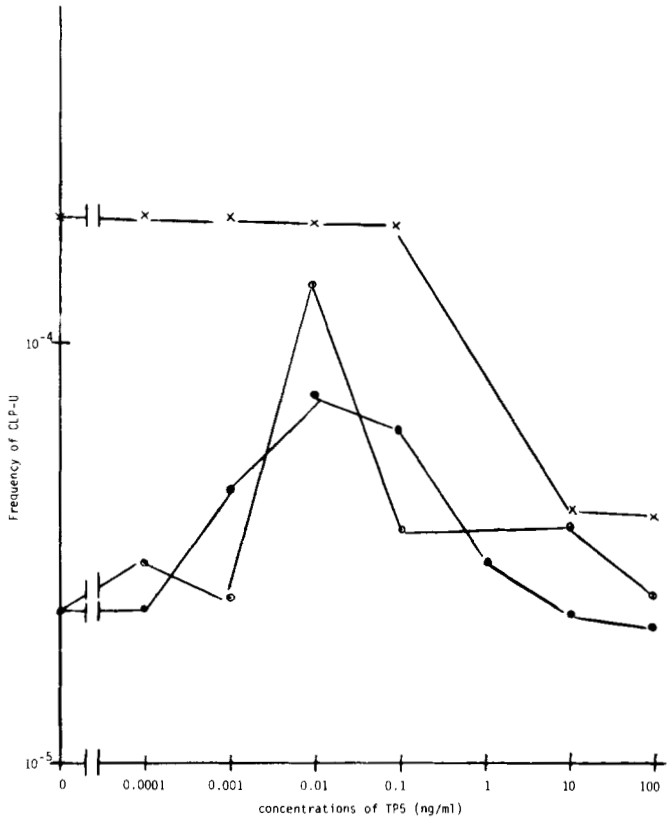

Figure 5. Graph showing the effect of TP5 *in vitro* on the frequency of CLP-U. Normal C57BL/6 spleen cells were incubated with different concentrations of TP5 for 1 hr at 37°C. The cells were washed and set up in limiting dilution assays as shown in Figures 1 and 2. As shown on the graph, 0.01 to 0.1 ng of TP5/ml enhances the frequency of CLP-U after suboptimal stimulation with 1.25×10^5 DBA cells (●) or 2.5×10^5 DBA cells (⊙). TP5, 10 to 100 ng, suppresses CLP-U enumeration after optimal stimulation with 5×10^5 DBA cells (×).

tory cells. These effects, which are detectable with *in vitro* and *in vivo* administration, are dose dependent and in broad terms may be described as enhancement of responsiveness to suboptimal stimulation, and, in higher doses, inhibition of responsiveness to supra-optimal doses. Bach (11) has also demonstrated that thymic serum factor is most effective in restoring normal immune response to thymectomized mice when suboptimal numbers of allogeneic cells were used. The immunoregulatory effect of TP5 has also been demonstrated in NZB mice (12) and there are preliminary data for immunoregulatory effects of TP5 in induced experimental autoimmune disease and experimental tumors (Lau and Goldstein, unpublished data). These findings suggest that lymphocyte subpopulations that are known to contain regulatory cells and control circuits of cell to cell communication (13, 14), may in turn be regulated by thymopoietin levels, a suggestion already hinted at by previous experiments (1). The rapid and persistent action of TP5 also opens the possibility of using this pentapeptide therapeutically to regulate the immune response.

Acknowledgments. The authors wish to thank Ms. Ulrike Kuprath for her excellent technical assistance and Ms. Linda Traeger and Ms. Dorothy S. Collins for preparing the manuscript.

REFERENCES

1. Weksler, M. E., J. B. Innes, and G. Goldstein. 1978. Immunological studies of aging. IV. The contribution of thymic involution to the immune deficiencies of aging mice and reversal with thymopoietin$_{32-36}$. J. Exp. Med. 148:996.
2. Goldstein, G., M. P. Scheid, E. A. Boyse, D. H. Schlesinger, and J. VanWauwe. 1979. A synthetic pentapeptide with biological activity characteristic of the thymic hormone thymopoietin. Science 204: 1309.
3. Basch, R. S., and G. Goldstein. 1974. Induction of T cell differentiation *in vitro* by thymin, a purified polypeptide hormone of the thymus. Proc. Natl. Acad. Sci. 71:1474.
4. Komuro, K., G. Goldstein, and E. A. Boyse. 1975. Thymus-repopulating capacity of cells that can be induced to differentiate to T cells *in vitro*. J. Immunol. 115:195.
5. Storrie, B., G. Goldstein, E. A. Boyse, and U. Hammerling. 1976. Differentiation of thymocytes: evidence that induction of the surface phenotype requires transcription and translation. J. Immunol. 116:1358.
6. Scheid, M. P., G. Goldstein, and E. A. Boyse. 1978. The generation and regulation of lymphocyte populations. Evidence from differentiative induction systems *in vitro*. J. Exp. Med. 147:1727.
7. Sunshine, G. H., R. S. Basch, R. G. Coffey, K. W. Cohen, G. Goldstein, and J. W. Hadden. 1978. Thymopoietin enhances the allogeneic response and cyclic GMP levels of mouse peripheral, thymus-derived lymphocytes. J. Immunol. 120:1594.
8. Teh, S. H., R. A. Phillips, and R. G. Miller. 1977. Quantitative studies on the precursors of cytotoxic lymphocytes. J. Immunol. 118:1049.
9. Lindahl, K. F., and D. B. Wilson. 1977. Histocompatible antigen-activated cytotoxic T lymphocytes. J. Exp. Med. 145:508.
10. Tischio, J. P., J. E. Patrick, H. S. Weintraub, M. Chasin, and G. Goldstein. 1979. Short *in vitro* half-life of thymopoietin$_{32-36}$ pentapeptide in human plasma. Int. J. Peptide Protein Res. 14:479.
11. Bach, M. A. 1977. Lymphocyte mediated cytotoxicity effects of aging, adult thymectomy and thymic factor. J. Immunol. 119:641.
12. Gershwin, M. E., W. Kruse, and G. Goldstein. 1979. The effect of thymopoietin$_{32-36}$ and ubiquitin on spontaneous immunopathology of New Zealand mice. J. Rheumatol. In press.
13. Eardley, D. D., J. Hugenberger, L. McVay-Boudreau, F. W. Shen, R. K. Gershon, and H. Cantor. 1978. Immunoregulatory circuits among T-cell sets. I. T-helper cells induce other T-cell sets to exert feedback inhibition. J. Exp. Med. 147:1106.
14. Cantor, H., L. McVay-Boudreau, J. Hugenberger, K. Nardorf, F. W. Shen, and R. K. Gershon. 1978. Immunoregulatory circuits among T-cell sets. II. Physiological role of feedback inhibition *in vivo*: absence in NZB mice. J. Exp. Med. 147:1116.